

**A TRANSMEMBRANE PROTEIN AS A DOWNSTREAM TARGET OF NEUROTROPHIN  
AND EPHRIN RECEPTOR TYROSINE KINASES, DNA ENCODING SAME AND  
MONOCLONAL ANTIBODIES THERETO**

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[0001] The experiments performed in this application were supported in part by the National Institutes of Health, Grant Nos. NS-21072, HD-23315, and NS-10489. The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of Grant Nos. NS-21072, HD-23315, and NS-10489 awarded by the National Institutes of Health.

**CROSS-REFERENCE TO RELATED APPLICATIONS**

[0002] The present application claims priority under 35 U.S.C. §119(e) from U.S. provisional application no. 60/256,909, filed December 21, 2000, the content of which is hereby incorporated entirely by reference.

**BACKGROUND OF THE INVENTION**

**Field of the Invention**

[0003] The present invention relates to a transmembrane protein which is a downstream target of neurotrophin and ephrin receptors for phosphorylation.

## Description of the Related Art

[0004] The formation of the nervous system requires appropriate connectivity of neurons and their targets both spatially and temporally. Two families of proteins that mediate these actions are the neurotrophins and ephrins. Neurotrophins play a prominent role in the development of the vertebrate nervous system by influencing cell survival, differentiation and cell death events (Levi-Montalcini, 1987; Lewin and Barde, 1996). Neurotrophins also exhibit acute regulatory effects upon neurotransmitter release, synaptic strength and connectivity (Thoenen, 1995; Bonhoeffer, 1996; McAllister et al., 1999). In addition to promoting axonal (Patel et al., 2000) and dendritic branching, neurotrophins serve as chemoattractants for extending growth cones *in vitro* (Letourneau, 1978; Gundersen and Barrett, 1979; Gallo et al., 1997).

[0005] Ephrins comprise another class of ligands that function in axon guidance, cell migration, axon fasciculation, boundary formation, topographic mapping, and morphogenesis (Frisen et al., 1999). Ephrins are a family of eight proteins that are found associated with the plasma membrane, either through a GPI linkage (as seen in the A subfamily) or as transmembrane proteins (as seen in the B subfamily). Ephrins signal via receptor protein tyrosine kinases, but the biological outcomes are distinct from mitogenic factors such as platelet-derived growth factor (PDGF)

and epidermal growth factor (EGF), both of which transmit signals through tyrosine phosphorylation (Bruckner and Klein, 1998; Flanagan and Vanderhaeghen, 1998; Holland et al., 1998). Ephrin receptor-associated molecules such as Crk, Nck, RasGAP, and Fyn are proposed links between the receptor and downstream events such as cell adhesion and cytoskeletal changes. In addition, Grb2, Grb10, and the p85 subunit of phosphatidylinositol-3-kinase (PI-3 kinase) are utilized in ephrin receptor signal transduction (Mellitzer et al., 2000).

[0006] The neurotrophins, NGF, BDNF, NT-3 and NT-4/5, exert their effects through two classes of receptors (Kaplan and Miller, 2000). TrkA, TrkB, and TrkC serve as receptors for NGF, BDNF and NT-4, and NT-3, respectively (Chao, 1992a). The p75 receptor is a member of the TNF receptor superfamily (Smith et al., 1994) and binds to all neurotrophins. Most central and peripheral neurons express p75 together with one or more of the Trks. The p75 receptor, when co-expressed with TrkA, provides a positive influence upon Trk function (Barker and Shooter, 1994; Verdi et al., 1994) and determines specificity of neurotrophin binding and responsiveness (Benedetti et al., 1994; Bibel et al., 1999; Brennan et al., 1999). An association of p75 and Trk receptors has been detected by co-precipitation (Huber and Chao, 1995; Gargano et al., 1997; Bibel et al., 1999) and co-patching

of these receptors has been observed using fluorescent labeled antibodies (Ross et al., 1996).

[0007] Receptor tyrosine kinases frequently utilize a number of common intracellular signaling components such as

5 phospholipase C- $\gamma$ , PI-3 kinase and adaptor proteins such as Shc and Grb2. Common to many of these proteins is their ability to bind to phosphorylated tyrosines via domains such as the SH2 and phosphotyrosine-binding (PTB) domains. How these shared signaling components lead to different biological outcomes is not  
10 well understood (Chao, 1992a). Possible mechanisms include receptor utilization of substrates in a differential manner (e.g., differential association/dissociation kinetics), competition for binding between different substrates (Meakin et al., 1999), or recruitment of unique target proteins, such as  
15 rAPS and SH2-B (Qian et al., 1998).

[0008] Citation of any document herein is not intended as an admission that such document is pertinent prior art, or considered material to the patentability of any claim of the present application. Any statement as to content or a date of  
20 any document is based on the information available to applicant at the time of filing and does not constitute an admission as to the correctness of such a statement.

# SUMMARY OF THE INVENTION

[0009] The present invention provides a polypeptide which is a transmembrane protein that is highly expressed in vertebrate central and peripheral nervous systems. This polypeptide, which associates with TrkA and p75 neurotrophin receptors, is a target for phosphorylation by neurotrophin and ephrin receptor tyrosine kinases, enhances neurotransmitter release, and modulates the clustering of proteins involved in ion channel formation, includes the amino acid sequence of: (A) SEQ ID NO:2; (B) SEQ ID NO:4; (C) a fragment of the polypeptide of SEQ ID NO:2; (D) a fragment of the polypeptide of SEQ ID NO:4; (E) a variant polypeptide which is at least 95% identical to SEQ ID NO:2; (F) a variant polypeptide which is at least 95% to SEQ ID NO:4; or (G) a functional derivative or a salt of (A), (B), (C), (D), (E), or (F).

[0010] The present invention also provides an isolated nucleic acid which encodes the polypeptide of the present invention or which hybridizes under stringent conditions to the complement of the nucleotide sequence encoding the polypeptide of the present invention a vector, a transformed host cell containing such a nucleic acid, and a method for producing the polypeptide of the present invention.

[0011] Further provided by the present invention is a molecule containing the antigen-binding portion of an antibody specific for the polypeptide of the present invention.

[0012] A still further aspect of the present invention  
5 provides a method for visualizing the growth cone of neurons, where the polypeptide of the present invention serves as a marker for growth cones.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

10 [0013] Figure 1 shows the predicted topology of ARMS. Transmembrane domains and various intracellular motifs are depicted.

[0014] Figure 2 shows the amino acid sequence and comparison of rat (SEQ ID NO:2) and human (SEQ ID NO:4) ARMS proteins.  
15 Dash-lined residues denote 11 contiguous ankyrin repeats; bold-faced tyrosine (Y) residues (at positions 399, 409, 441, 444, and 466 of the rat sequence) are evolutionarily conserved among human, rat, *Drosophila*, and *C. elegans*; boxed residues are the putative transmembrane domains; italicized residues denote the  
20 polyproline stretch; shadowed residues constitute the SAM domain (aa1152-1221); carboxy-most three asterisked amino acids (SIL) encode a PDZ-binding motif.

[0015] Figure 3 shows the comparison of various cytoplasmic regions of rat (r), human (h), *Drosophila* (d) and *C. elegans* (w)

[0016] Figures 4A and 4B show a Northern analysis of ARMS (Fig. 4A) and a methylene blue staining of the 28S ribosomal band as a loading control (Fig. 4B). A single transcript of 7.0 kb was detected by Northern analysis using a <sup>32</sup>P-labeled ARMS cDNA probe (Fig. 4A). Each lane contained 20 µg of total RNA (with the exception of pancreas and DRG lanes which contained <10 µg each) extracted from various rat tissue.

[0017] Figures 5A-5D show the distribution of ARMS mRNA in the adult rat central nervous system by *in situ* hybridization. A <sup>33</sup>P-labeled cRNA probe was used to assess ARMS mRNA expression. Areas of intense labeling include the mitral cell layer of the

olfactory bulb (OB; Fig. 5A), all regions of the hippocampus (HP; Fig. 5B), the Purkinje cell layer of the cerebellum (CB; Fig. 5C), grey matter -- most notably in the ventral horn -- of the spinal cord (SC; Fig. 5D).

5 [0018] Figures 6A and 6B show the expression in adult rat dorsal root ganglion (DRG) by *in situ* hybridization. A <sup>33</sup>P-labeled cRNA probe was used to assess mRNA distribution in DRG as depicted in the dark field image (left). The majority of cell bodies of the DRG were positive for ARMS mRNA expression, but  
10 notable absences of expression were localized to the large diameter DRG cell bodies as depicted by the arrows in the dark field and the corresponding phase (Fig. 6B) photographs. (scale bars = 1 mm)

[0019] Figures 7A-7C show expression of ARMS mRNA by *in situ*  
15 hybridization of ARMS in embryonic day 14 (E14) rat. In a coronal section through the midsection of an E14 rat, only spinal cord (sc) and dorsal root ganglion (drg) were positive for ARMS (Fig. 7A). *In situ* hybridization of ARMS in a midsagittal section (Fig. 7B) and a more lateral section (Fig. 7C) of E14  
20 rat. ARMS mRNA expression was restricted to various brain regions such as the cortex (cx), hippocampus (hp), pons, medulla (med), basal telencephalon (bt), principal and spinal trigeminal nucleus (tn), superior and inferior colliculus (clc) and spinal cord (sc). Multiple ganglia expressed ARMS mRNA, such as the



dorsal root ganglion (drg), trigeminal ganglion (tg), geniculate ganglion (gg), vestibular ganglion (vg), and superior cervical ganglion (scg). (white scale bars = 1 mm; black scale bar = 50  $\mu$ m)

5 [0020] Figures 8A-8B show the interaction of p75 with ARMS. HEK293T cells were co-transfected with cDNAs encoding full length ARMS, HA-tagged p75, ARMS plus p75, or empty vector. Cells lysates were immunoprecipitated with anti-ARMS 892 antiserum and immunoblotted with anti-HA (Fig. 8A). Expression of p75  
10 receptors was confirmed by immunoblotting with anti-p75 (9992; Fig. 8B).

[0021] Figures 9A-9D show coprecipitation of TrkA and ARMS (Fig. 9A) and colocalization of TrkA and ARMS (Figs. 9B-9D). In Fig. 9A, PC12 615 cells were treated for 10 minutes and 25 hours  
15 with NGF (100 ng/ml). Lysates were prepared and subjected to immunoprecipitation with anti-Trk C-14 antibody, followed by immunoblot with anti-ARMS antibody. Normal rabbit IgG was used as a negative control. In Figs. 9B-9D, immunofluorescence analysis of ARMS and TrkA receptor in sympathetic neurons is  
20 shown. SCG sympathetic neurons were grown in the presence of 150 ng/ml NGF, fixed and immunostained as described in the Methods section of Example 1. The ARMS protein and the TrkA receptor were subjected to double immunostaining using an anti-ARMS antiserum (Fig. 9B) and an anti-Trk B-3 (Fig. 9C) monoclonal

antibody and analyzed by confocal microscopy. The signal observed in Fig. 9D demonstrates overlap of the two signals (overlay) from immunostaining with anti-ARMS anti-serum and with an anti-TrkB monoclonal antibody. The arrow indicates cell surface co-localization of ARMS and TrkA.

[0022] Figures 10A and 10B show tyrosine phosphorylation of ARMS. In Fig. 10A, phosphorylation of ARMS by NGF in PC12 cells is rapid and can be blocked by K252a. The antiserum 892 was used to immunoprecipitate endogenously expressed ARMS from PC12 cell lysates. Anti-phosphotyrosine antibody, pY99 was used to assess tyrosine phosphorylation of the immunoprecipitated ARMS. Within 1 minute of NGF treatment, phosphorylation of ARMS could be detected, suggesting a direct phosphorylation by TrkA. Furthermore, 100 nM K252a potently blocked ARMS phosphorylation (top). In lysates of the same samples, TrkA autophosphorylation is shown using pY99 (bottom). The time course of ARMS phosphorylation by NGF in PC12 cells is shown in Fig. 10B. The phosphorylation peaks within 10 minutes (m) and is sustained for at least 25 hours (h) (top). Reprobing of the same blot with 892 demonstrated equivalent levels of immunoprecipitated ARMS from the various lysates (bottom).

[0023] Figure 11 shows specificity of ARMS phosphorylation. Phosphorylation of ARMS is specifically induced upon NGF, but not EGF, treatment of PC12 615 cells. Two time points, 10 minutes

and 2 hours, were examined for tyrosine phosphorylation of ARMS using the following conditions: no ligand (CTRL), 50 ng/ml EGF and 100 ng/ml NGF. To demonstrate the specificity of the ARMS antiserum, 892 (I), preimmune antiserum (P) was used in parallel immunoprecipitations (IP). The lower panel shows the amount of ARMS protein that was immunoprecipitated from the various lysates.

[0024] Figure 12 shows the effects of other neurotrophins. The neurotrophins BDNF and NT-4/5 induce phosphorylation of ARMS through the TrkB receptor. PC12 cells stably expressing TrkB were treated with either 100 ng/ml BDNF or 100 ng/ml NT4/5 and the phosphorylation of ARMS was measured as described in Figure 10A. BDNF, and to a lesser extent, NT4/5, were able to induce tyrosine phosphorylation of ARMS. The bottom panel depicts immunoprecipitated ARMS from each lysate.

[0025] Figure 13 shows induction of ARMS phosphorylation in hippocampal neurons by BDNF. Primary cultures of E17 hippocampal neurons were prepared and treated with 50 ng/ml BDNF for the indicated times. Phosphorylation of ARMS was assessed by immunoprecipitation with anti-ARMS 892 antiserum and Western blotting with anti-phosphotyrosine pY99 antibody (top panel). Equal amounts of ARMS protein were immunoprecipitated from each lysate as shown with reprobing the same blot with 892.

[0026] Figures 14A and 14B show the effects of ephrins.

In Fig. 14A, ephrin B2 induces ARMS tyrosine phosphorylation in NG108-15 cells expressing EphB2 receptor. Lysates were made from untreated or ligand-stimulated NG108-15 cells (using aggregated ephrin B2, 30-40 minutes) and immunoprecipitated with 892  
5 antiserum. Tyrosine phosphorylation was assessed with pY99 in subsequent Western blots. Equivalent amounts of ARMS were immunoprecipitated as shown in the lower panel. In Fig. 14B, tyrosine phosphorylation of ARMS by ephrin B2 peaks at 30 minutes. Thus, the time course of ARMS tyrosine phosphorylation  
10 closely parallels that of receptor autophosphorylation.

[0027] Figures 15A and 15B show ARMS/Trk receptor interaction. Expression plasmids containing full length cDNAs for ARMS and TrkA, TrkB and TrkC receptors were transiently transfected into HEK293 cells ( $2 \times 10^6$  cells/plate) following the calcium-  
15 phosphate method. ARMS, Trk and EGF receptor expression were detected by immunoblotting. Cells were lysed in 1% NP-40 lysis buffer containing 20 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40, 2 mM EDTA and protease inhibitors (0.15 units/ml aprotinin, 20  $\mu$ M leupeptin and 1 mM phenylmethylsulphonylfluoride), at 4°C, for 30  
20 min. Immunoprecipitation was performed for 3 hours at 4°C using 2-3 mg of total protein extract and the Flag agarose-conjugated antibody (Sigma, St. Louis, MO). After several washes, immunoprecipitates were analyzed by SDS-PAGE followed by Western blot with different antibodies e.g., antibodies against TrkA,

TrkB, TrkC, Arms, or EGFR. Reactive protein bands were visualized by enhanced chemiluminescence detection (Amersham Corp., Piscataway, NJ).

[0028] Figures 16A and 16B show a PC12 Immunofluorescence analysis. PC12 cells with (Fig. 16A) or without (Fig. 16B) NGF treatment cultured in Lab-Tek chamber slides (Nalge Nunc International) coated with collagen and poly-L-lysine were fixed with paraformaldehyde and permeablized with cold methanol. Cells were blocked with PBS containing 10% FCS and incubated with purified antibody (3 µg/ml) against the C-terminus of ARMS protein, 892 (Example 1). Primary antibodies were visualized using fluorescence-conjugated secondary antibodies (FITC-conjugated goat anti-rabbit IgG; Jackson Laboratories). Images were collected on a Leica confocal microscope (Nussloch, Germany) and show that ARMS is localized at neurite tips in PC12 cells after NGF treatment.

[0029] Figures 17A-17F show immunolocalization of ARMS and VAMP-2, a synaptic vesicle marker, in hippocampal neurons. Primary cultures of hippocampal neurons were obtained from rats E17-19 and maintained with Neurobasal medium (Gibco) supplemented with B-27 and 0.4 mM glutamine in Lab-Tek chamber slides (Nalge Nunc International) coated with poly-L-lysine. The cells were fixed with paraformaldehyde, permeablized with cold methanol and then blocked with PBS containing 10% FBS, 10% normal goat serum

and 5% BSA for at least 30 minutes and incubated with anti-ARMS antibody 892 (Fig. 17A) and VAMP-2 (Fig. 17B) antibodies in blocking solution at room temperature or 4°C. Primary antibodies were detected using fluorescence-conjugated secondary antibodies (FITC-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG (Jackson Laboratories)). Images were collected on a Leica confocal microscope. Fig. 17C is a merged image of Figs. 17A and 17B). Figs. 17D-17F represent an enlargement of the staining in the top Figs. 17A-17C,

respectively, with the white arrows designating tips of processes

[0030] Figure 18 shows the localization of ARMS in axons and growth cones of hippocampal neurons. Cultures of hippocampal neurons were assessed for ARMS expression by indirect immunofluorescence, as described above for Fig. 17A. A concentration of ARMS protein was found at the growth cone (white arrow) and along the axon in a punctate distribution.

[0031] Figure 19 shows the interaction between ARMS and PDZ-containing proteins GRIP1, GRIP2 and PICK1. Expression plasmids containing myc-epitope tagged GRIP1, GRIP2 or PICK1 were cotransfected with a full length cDNA for ARMS in HEK293 cells. Cells were lysed in 1% NP-40 lysis buffer and immunoprecipitation was carried out with anti-myc antibodies, followed by Western blot for the ARMS protein.

[0032] Figure 20 shows a proposed schematic model of interactions between Trk receptors, ARMS, PDZ-containing proteins and glutamate receptors.

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# **DETAILED DESCRIPTION OF THE INVENTION**

[0033] The present inventors have discovered a novel transmembrane protein, designated ARMS (for Ankyrin Repeat-Rich, Membrane Spanning), in rats and humans which is a downstream target/substrate of neurotrophin and ephrin receptor tyrosine  
10 kinases, Trk and Eph, respectively. This ARMS protein/  
polypeptide according to the present invention was found to have 1,715 amino acids (SEQ ID NO:2 for rat ARMS and SEQ ID NO:4 for human ARMS) containing four putative transmembrane domains, multiple ankyrin repeats, a SAM domain, and a potential PDZ-  
15 binding motif.

[0034] The ARMS protein of the present invention is a substrate for tyrosine phosphorylation by the Trk neurotrophin family of tyrosine kinase receptors. These receptors mediate the actions of neurotrophins (NGF, BDNF, NT-3 and NT-4), which are  
20 required for the survival and differentiation of neurons during development and after injury. Moreover, neurotrophin receptor signaling is required for a form of memory known as long term potentiation (LTP), formation of synapses, axonal targeting and hyperalgesia (pain). The ARMS protein is specifically a target

for neurotrophins and ephrins, which are also responsible for axon guidance. Unlike other phosphotyrosine proteins, the ARMS protein is a specific target for neurotrophins and ephrins, and not for other polypeptide growth factors which use tyrosine  
5 kinase receptors.

[0035] Because of its specificity and highly neuronal expression pattern, ARMS is a useful indicator of the biological activity of neurotrophins and ephrins. The ARMS gene is expressed in postmitotic neurons during the stage of development  
10 in which extensive axon pathfinding is occurring and is also expressed during adulthood in highly "plastic" regions of the brain such as the hippocampus, cortex, Purkinje cells of the cerebellum, the olfactory bulb, and of the spinal cord motor neurons, regions which are enriched in Trk and Eph receptors.

[0036] The most significant commonality of these neuronal  
15 populations is their ability to undergo continued synaptic changes throughout adult life. Neurons of the olfactory bulb are continually renewed and hence must form new synapses; hippocampal neurons can undergo synaptic remodeling and LTP; Purkinje cell  
20 dendrites are highly plastic due to their constant structural remodeling; and motor neurons have the capacity to regenerate and to form new synapses with peripheral targets in adults. Peripheral neurons have regenerative properties throughout adulthood, and this process requires axon outgrowth and new



synapse formation. Neurotrophins and ephrins are likely candidates in spinal cord regeneration (Frisen et al., 1992; Miranda et al., 1999), and the present inventors believe that ARMS is also utilized in this process.

5 [0037] The ARMS gene is also expressed in sensory, geniculate, vestibular and sympathetic neurons in the peripheral nervous system. These neural regions control hearing, vision, taste, smell, cardiovascular and motor functions. The ARMS protein is a very abundant neuronal-specific protein that contains consensus  
10 docking sites for ion channels and other signal transduction proteins. From immunofluorescence and confocal microscopic studies, there is a dramatic change in the distribution of ARMS in both hippocampal neurons and PC12 cells after treatment with neurotrophins BDNF or NGF, demonstrating that the ARMS protein is  
15 localized discretely at growth cones and in synaptic regions of neuronal membranes where neuronal activities are located.

[0038] The predicted integral membrane structure of ARMS led the present inventors to believe that ARMS may function as an ion channel. The four predicted transmembrane domains of the  
20 ARMS protein and its overall structure is reminiscent of the TRP family of ion channels and the capsaicin receptor, VR1 (Caterina et al., 1997; Harteneck et al., 2000). These channels contain six transmembrane domains with intracellular amino and carboxy termini and amino terminal ankyrin repeats. Although ARMS

channel activity was not detected in gene transfer experiments, it remains possible that this protein may also serve as a subunit of a channel, or more likely, is involved in clustering or maintenance of ion channels. Such a possibility has been suggested by the ability of the neurotrophin BDNF to elicit hippocampal, cortical, and cerebellar depolarization on a very rapid time scale (Kafitz et al., 1999), presumably through activation of sodium channels. While the mechanism of this activation is unknown, phosphorylation of ARMS by TrkB suggests a role for ARMS in neurotrophin-mediated regulation of neuronal activity, such as changes in electrophysiological activity of neurons.

[0039] Furthermore, the presence of several protein interaction domains strongly supports a role for ARMS in recruiting proteins to Trk receptor tyrosine kinases. Indeed, co-immunoprecipitation studies indicate that ARMS may be an integral part of a higher order Trk-p75 receptor complex. These interactions may not be limited to neurotrophin signaling, as the Eph receptor family is also capable of phosphorylating the ARMS protein.

[0040] Several neuronal proteins were found to bind to the ARMS protein. These proteins include synembryn, synaptotagmin,  $\alpha$ -catenin and cdcRel, which are all involved in synaptic vesicle trafficking. ARMS appears to be involved in the neurotransmitter

vesicle movements in the nerve cells and may be involved in directing the release of neurotransmitters from neurons. These neurotransmitters may be acetylcholine or dopamine.

Another protein that was discovered to interact with the ARMS protein is the regulatory subunit of  $K^+$  channels. This indicates that ARMS may be associated with ion channel activities, which give rise to changes in neuronal activity (action potentials). The significance of these findings stem from the potent effects of neurotrophins upon synaptic plasticity. The phosphorylation of ARMS by Trk and Eph receptors provides a mechanism for transmitting information from neurotrophins to specific regions of synaptic activity in nerve cells.

[0041] One aspect of the present invention is therefore directed to an ARMS polypeptide which associates with TrkA and p75 neurotrophin receptors and is a target for phosphorylation by neurotrophin and ephrin receptor tyrosine kinases. This polypeptide of the present invention contains the rat ARMS amino acid sequence of SEQ ID NO:2, a fragment of the polypeptide of SEQ ID NO:2 or a variant thereof which is at least 95% identical to SEQ ID NO:2, the human ARMS amino acid sequence of SEQ ID NO:4, a fragment of the polypeptide of SEQ ID NO:4 or a variant thereof which is at least 95% identical to SEQ ID NO:4, or a derivative or salt of any of the above.

[0042] Fragments of the full-length ARMS polypeptide of SEQ ID NO:2 or SEQ ID NO:4 are intended to cover any active fragment that retains the biological activity of the full-length ARMS polypeptide. For example, fragments can be readily generated from the full-length ARMS polypeptide where successive residues can be removed from either or both the N-terminus or C-terminus of ARMS, or from peptides obtained thereof by enzymatic or chemical cleavage of the polypeptide. Thus, multiple substitutions are not involved in screening for active fragments of ARMS. If the removal of one or two amino acids from one end or the other does not affect the biological activity after testing in assays described in the Examples and Figures herein, such truncated polypeptides are considered to be within the scope of the present invention. Further truncations can then be carried out until it is found where the removal of another residue destroys the biological activity.

[0043] Preferably, such active fragments of ARMS would contain the PDZ-binding motif in the cytoplasmic C-terminal portion of ARMS and further either contain one or more transmembrane domains of ARMS or be fused as a fusion polypeptide to a transmembrane domain, other than a transmembrane domain of ARMS, which allows a fragment of ARMS to retain the biological activity of the full-length ARMS polypeptide.

[0044] The term "variant" is intended to encompass a variant of the native sequence of SEQ ID NO:2 or SEQ ID NO:4, or of a biologically active fragment thereof, which has an amino acid sequence having at least 85% identity, preferably at least 90% identity, or more preferably at least 95% identity to the native sequence and retains the biological activity thereof. As would be appreciated by those of skill in the art, this term covers naturally-occurring variants as a preferred embodiment.

[0045] "Functional derivatives" as used herein covers chemical derivatives which may be prepared from the functional groups which occur as side chains on the residues or the N- or C-terminal groups, by means known in the art, and are included in the invention as long as they do not destroy the biological activity of the corresponding polypeptide/protein as described herein. Derivatives may have chemical moieties, such as carbohydrate or phosphate residues, provided such derivative have the same biological activity.

[0046] Suitable derivatives may include aliphatic esters of the carboxyl groups, amides of the carboxyl groups by reaction with ammonia or with primary or secondary amines, N-acyl derivatives or free amino groups of the amino acid residues formed with acyl moieties (e.g., alkanoyl or carbocyclic aroyl groups), O-acyl derivatives of free hydroxyl group (e.g., that of seryl or threonyl residues) formed with acyl moieties, or

phosphorylated derivatives of free hydroxyl groups in serine and threonine residues. Such derivatives may also include for example, polyethylene glycol side-chains which may mask antigenic sites and extend the residence of the complex or the portions thereof in body fluids.

[0047] Non-limiting examples of such derivatives are described below.

[0048] Cysteiny l residues most commonly are reacted with alpha-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteiny l residues also are derivatized by reaction with bromotrifluoroacetone, alpha-bromo-beta-(5-imidazolyl)propionic acid, chloroacetyl phosphate, -alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl-2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

[0049] Histidyl residues are derivatized by reaction with diethylprocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain.

Parabromophenacyl bromide also is useful; the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

[0050] Lysiny l and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the

lysinyl residues. Other suitable reagents for derivatizing  
alpha-amino-containing residues include imidoesters such as  
methyl picolinimide; pyridoxal phosphate; pyridoxal;  
chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea;  
5 2, 4-pentanedione; and transaminase-catalyzed reaction with  
glyoxylate.

[0051] Arginyl residues are modified by reaction with one or  
several conventional reagents, among them phenylglyoxal, 2,3-  
butanedione, 1,2-cyclodexanedione, and ninhydrin. Derivatization  
10 of arginine residues requires that the reaction be performed in  
alkaline conditions because of the high  $pK_a$  of the guanidine  
functional group. Furthermore, these reagents may react with the  
groups of lysine as well as the arginine epsilon-amino group.

[0052] The specific modification of tyrosyl residues *per se*  
15 has been studied extensively, with particular interest in  
introducing spectral labels into tyrosyl residues by reaction  
with aromatic diazonium compounds or tetranitromethane. Most  
commonly, N-acetylimidazole and tetranitromethane are used to  
form O-acetyl tyrosyl species and 3-nitro derivatives,  
20 respectively.

[0053] Carboxyl side groups (aspartyl or glutamyl) are  
selectively modified by reaction with carbodiimides ( $R'-N-C-N-R'$ )  
such as 1-cyclohexyl-3-[2-morpholinyl-(4-ethyl)]carbodiimide or  
1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide.

Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

[0054] Glutaminyl and asparaginyl residues are frequently  
5 deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

[0055] The term "derivatives" is intended to include only  
10 those derivatives that do not change one amino acid to another of the twenty commonly-occurring natural amino acids.

[0056] The term "salts" herein refers to both salts of carboxyl groups and to acid addition salts of amino groups of the complex of the invention or analogs thereof. Salts of a carboxyl  
15 group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases as those formed, for example, with amines, such as triethanolamine, arginine or lysine, piperidine, procaine and the like. Acid  
20 addition salts include, for example, salts with mineral acids, such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids, such as, for example, acetic acid or oxalic acid. Of course, any such salts must have substantially similar biological activity to the polypeptide of the invention.



[0057] Another aspect of the present invention is directed to a molecule which contains the antigen-binding portion of an antibody specific for the ARMS polypeptide of the present invention. It should be understood that the term "antibody" is intended to include intact antibodies, such as polyclonal antibodies or monoclonal antibodies (mAbs), as well as proteolytic fragments thereof such as the Fab or F(ab')<sub>2</sub> fragments. Furthermore, the DNA encoding the variable region of the antibody can be inserted into DNA encoding other antibodies to produce chimeric antibodies (see, for example, U.S. Patent 4,816,567).

[0058] Single chain antibodies can also be produced and used. Single chain antibodies can be single chain composite polypeptides having antigen binding capabilities and comprising a pair of amino acid sequences homologous or analogous to the variable regions of an immunoglobulin light and heavy chain (linked V<sub>H</sub>-V<sub>L</sub> or single chain F<sub>V</sub>). Both V<sub>H</sub> and V<sub>L</sub> may copy natural monoclonal antibody sequences or one or both of the chains may comprise a CDR-FR construct of the type described in U.S. Patent 5,091,513 (the entire contents of which are hereby incorporated herein by reference). The separate polypeptides analogous to the variable regions of the light and heavy chains are held together by a polypeptide linker. Methods of production of such single chain antibodies, particularly where the DNA

encoding the polypeptide structures of the  $V_H$  and  $V_L$  chains are known, may be accomplished in accordance with the methods described, for example, in U.S. Patents 4,946,778, 5,091,513 and 5,096,815, the entire contents of each of which are hereby  
5 incorporated herein by reference.

[0059] A "molecule which contains the antigen-binding portion of an antibody" is intended to include not only intact immunoglobulin molecules of any isotype and generated by any animal cell line or microorganism, or generated *in vitro*, such as  
10 by phage display technology for constructing recombinant antibodies, but also the antigen-binding reactive fraction thereof, including, but not limited to, the Fab fragment, the Fab' fragment, the  $F(ab')_2$  fragment, the variable portion of the heavy and/or light chains thereof, and chimeric or single-chain  
15 antibodies incorporating such reactive fraction, as well as any other type of molecule or cell in which such antibody reactive fraction has been physically inserted, or molecules developed to deliver therapeutic moieties by means of a portion of the molecule containing such a reactive fraction. Such molecules may  
20 be provided by any known technique, including, but not limited to, enzymatic cleavage, peptide synthesis or recombinant techniques.

[0060] The polypeptide of the present invention can be used as a marker for neuronal cells which have the ability to undergo

continued synaptic changes through adult life or for the presence and distribution (i.e, clustering and aggregation) of the ARMS polypeptide in such neuronal cells. More specifically, as demonstrated in Example 2, the polypeptide of the present invention is a marker for growth cones as ARMS was found to be localized discretely at growth cones and in the synaptic regions of neurons. It was also found that ARMS co-localized with Vamp-2, a synaptic vesicle marker, and can therefore be used for the same purposes as Vamp-2.

[0061] A molecule which contains the antigen-binding portion of an antibody specific for the ARMS polypeptide of the present invention can be labeled with a labeling compound and imaged *in vitro* or *in vivo* as is well-known in the diagnostic and imaging art. As a preferred embodiment, this molecule can be used in a method for visualizing the growth cone of neurons which method involves contacting a molecule containing the antigen-binding portion of an antibody specific for the ARMS polypeptide of the present invention with neurons to detect the presence of the polypeptide of SEQ ID NO:2 or SEQ ID NO:4 as a marker for the growth cone of neurons. The growth cone of neurons can then be visualized when the molecule containing the antigen-binding portion of an anti-ARMS antibody is detectably labeled, either directly or indirectly, i.e., through the binding of a secondary detectably labeled antibody to the anti-ARMS antibody.

[0062] Further aspects of the present invention is directed to a nucleic acid or polynucleotide which encodes the ARMS polypeptide of the present invention, a vector containing the nucleic acid, and a host cell transformed with such a nucleic acid. Preferably, the nucleic acid includes the nucleotide sequence encoding either the rat ARMS polypeptide of SEQ ID NO:2 or the human ARMS polypeptide of SEQ ID NO:4 or fragments thereof. More preferably, the nucleic acid includes the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:3.

[0063] The nucleic acid according to the present invention is intended to encompass nucleic acids which specifically hybridizes under stringent conditions to the complement of either SEQ ID NO:1 or SEQ ID NO:3. Stringency conditions are a function of the temperature used in the hybridization experiment and washes, the molarity of the monovalent cations in the hybridization solution and in the wash solution(s) and the percentage of formamide in the hybridization solution. In general, sensitivity by hybridization with a probe is affected by the amount and specific activity of the probe, the amount of the target nucleic acid, the detectability of the label, the rate of hybridization, and the duration of the hybridization. The hybridization rate is maximized at a  $T_i$  (incubation temperature) of 20-25°C below  $T_m$  for DNA:DNA hybrids and 10-15°C below  $T_m$  for DNA:RNA hybrids. It is also maximized by an ionic strength of about 1.5M  $Na^+$ . The

rate is directly proportional to duplex length and inversely proportional to the degree of mismatching.

[0064] Specificity in hybridization, however, is a function of the difference in stability between the desired hybrid and "background" hybrids. Hybrid stability is a function of duplex length, base composition, ionic strength, mismatching, and destabilizing agents (if any).

[0065] The  $T_m$  of a perfect hybrid may be estimated for DNA:DNA hybrids using the equation of Meinkoth et al (1984), as

$$T_m = 81.5^{\circ}\text{C} + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% \text{ form}) - 500/L$$

and for DNA:RNA hybrids, as

$$T_m = 79.8^{\circ}\text{C} + 18.5 (\log M) + 0.58 (\%GC) - 11.8 (\%GC)^2 - 0.56(\% \text{ form}) - 820/L$$

where

M, molarity of monovalent cations, 0.01-0.4 M NaCl,

%GC, percentage of G and C nucleotides in DNA, 30%-75%,

% form, percentage formamide in hybridization solution,

and

L, length hybrid in base pairs.

[0066]  $T_m$  is reduced by 0.5-1.5°C (an average of 1°C can be used for ease of calculation) for each 1% mismatching.

[0067] The  $T_m$  may also be determined experimentally. As increasing length of the hybrid (L) in the above equations

increases the  $T_m$  and enhances stability, the full-length rat gene sequence can be used as the probe.

[0068] Filter hybridization is typically carried out at 68°C, and at high ionic strength (e.g., 5 - 6 X SSC), which is non-  
 5 stringent, and followed by one or more washes of increasing stringency, the last one being of the ultimately desired high stringency. The equations for  $T_m$  can be used to estimate the appropriate  $T_i$  for the final wash, or the  $T_m$  of the perfect duplex can be determined experimentally and  $T_i$  then adjusted  
 10 accordingly.

[0069] Hybridization conditions should be chosen so as to permit allelic variations, but avoid hybridizing to other genes. In general, stringent conditions are considered to be a  $T_i$  of 5°C below the  $T_m$  of a perfect duplex, and a 1% divergence corresponds  
 15 to a 0.5-1.5°C reduction in  $T_m$ . Typically, rat clones were 95-100% identical to database rat sequences, and the observed sequence divergence may be artifactual (sequencing error) or real (allelic variation). Hence, use of a  $T_i$  of 5-15°C below, more preferably 5-10°C below, the  $T_m$  of the double stranded form of  
 20 the probe is recommended for probing a rat cDNA library with a rat DNA probe or a human cDNA library with a human DNA probe.

[0070] As used herein, highly stringent conditions are those which are tolerant of up to about 5% sequence divergence. Without limitation, examples of highly stringent (10°C below the

calculated  $T_m$  of the hybrid) conditions use a wash solution of 0.1 X SSC (standard saline citrate) and 0.5% SDS at the appropriate  $T_i$  below the calculated  $T_m$  of the hybrid. The ultimate stringency of the conditions is primarily due to the washing conditions, particularly if the hybridization conditions used are those which allow less stable hybrids to form along with stable hybrids. The wash conditions at higher stringency then remove the less stable hybrids. A common hybridization condition that can be used with the highly stringent wash conditions described above is hybridization in a solution of 6 X SSC (or 6 X SSPE), 5 X Denhardt's reagent, 0.5% SDS, 100  $\mu$ g/ml denatured, fragmented salmon sperm DNA at an appropriate incubation temperature  $T_i$ .

[0071] A still further aspect of the present invention is directed to a method for producing a polypeptide of the present invention by culturing in a nutrient medium a host cell transformed with the nucleic acid according to the present invention, as discussed above, to produce and express the polypeptide and then recovering the produced polypeptide.

[0072] Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration and is not intended to be limiting of the present invention.

1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100	2101	2102	2103	2104	2105	2106	2107	2108	2109	2110	2111	2112	2113	2114	2115	2116	2117	2118	2119	2120	2121	2122	2123	2124	2125	2126	2127	2128	2129	2130	2131	2132	2133	2134	2135	2136	2137	2138	2139	2140	2141	2142	2143	2144	2145	2146	2147	2148	2149	2150	2151	2152	2153	2154	2155	2156	2157	2158	2159	2160	2161	2162	2163	2164	2165	2166	2167	2168	2169	2170	2171	2172	2173	2174	2175	2176	2177	2178	2179	2180	2181	2182	2183	2184	2185	2186	2187	2188	2189	2190	2191	2192	2193	2194	2195	2196	2197	2198	2199	2200	2201	2202	2203	2204	2205	2206	2207	2208	2209	2210	2211	2212	2213	2214	2215	2216	2217	2218	2219	2220	2221	2222	2223	2224	2225	2226	2227	2228	2229	2230	2231	2232	2233	2234	2235	2236	2237	2238	2239	2240	2241	2242	2243	2244	2245	2246	2247	2248	2249	2250	2251	2252	2253	2254	2255	2256	2257	2258	2259	2260	2261	2262	2263	2264	2265	2266	2267	2268	2269	2270	2271	2272	2273	2274	2275	2276	2277	2278	2279	2280	2281	2282	2283	2284	2285	2286	2287	2288	2289	2290	2291	2292	2293	2294	2295	2296	2297	2298	2299	2300	2301	2302	2303	2304	2305	2306	2307	2308	2309	2310	2311	2312	2313	2314	2315	2316	2317	2318	2319	2320	2321	2322	2323	2324	2325	2326	2327	2328	2329	2330	2331	2332	2333	2334	2335	2336	2337	2338	2339	2340	2341	2342	2343	2344	2345	2346	2347	2348	2349	2350	2351	2352	2353	2354	2355	2356	2357	2358	2359	2360	2361	2362	2363	2364	2365	2366	2367	2368	2369	2370	2371	2372	2373	2374	2375	2376	2377	2378</
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## MATERIALS AND METHODS

### Materials

[0074] NGF was obtained from Harlan (Indianapolis, IN); BDNF from PeproTech (Rocky Hill, NJ); NT4/5 was a generous gift of Regeneron (Tarrytown, NY); EGF was obtained from Intergen (Purchase, NY); and K252a from Calbiochem (La Jolla, CA).

### Construction of two-hybrid DRG library and yeast two-hybrid screen

[0075] A cDNA library was constructed into unique Bst XI/Not I sites of a modified version of pJG4-5. Briefly, polyadenylated RNA, which had been purified from adult mouse and P1 rat DRG using Trizol (Gibco, Gaithersburg, MD) and the PolyA Tract System (Promega, Madison, WI), was used as template for reverse transcription (Gibco, Gaithersburg, MD) with an oligo-dT/Not I primer. Subsequent ligation into pJG4-5 and electroporation into DH5 $\alpha$  yielded a library of approximately  $10^6$  cfu and average insert size of 1.5-2.0 kilobases. A two-hybrid interaction screen, based on the LexA system (Gyuris et al., 1993), was performed in EGY48. The bait consisted of the entire cytoplasmic region of rat p75 as an in-frame fusion with the LexA DNA binding domain. Library cDNAs were expressed as in-frame fusions with the Gal4 transcriptional activation domain. Approximately 90 million yeast transformants were screened for their ability to survive in the absence of leucine. Sequence analysis identified

a novel cDNA clone of approximately 2.5 kb that corresponded to the carboxy terminal 250 amino acids of ARMS.

## Isolation of ARMS cDNA

5        [0076] Several rat brain libraries (young adult whole brain, adult whole brain and adult hippocampus) were screened to obtain the full-length ARMS cDNA. Criteria used to establish the initiator methionine include the following: assessment of multiple, independent cDNA fragments spanning the start site, an upstream, in-frame stop codon, and conformity to the Kozak consensus sequence.

### Northern blotting and *in situ* hybridization

[0077] Total RNA was extracted from various rat tissues using Trizol reagent (Gibco, Gaithersburg, MD). Twenty micrograms of total RNA were loaded per lane (with the exception of pancreas and DRG RNAs, which were <10 µg per lane), electrophoresed through a 2.2M formaldehyde/1% agarose gel, transferred to nylon membrane (Qiagen, Santa Clarita, CA), baked for 2 hours at 80°C, and probed with a cDNA fragment of ARMS labelled with <sup>32</sup>P-dCTP using Ready-To-Go (Amersham Pharmacia Biotech, Piscataway, NJ). *In situ* hybridization was performed as previously described (Lai and Lemke, 1991). Briefly, 30 µm paraformaldehyde-fixed brain sections from adult rat or whole embryos were slide-mounted,

hybridized with a  $^{32}\text{P}$ -labelled cRNA probe generated by T7 RNA polymerase from a PCR-generated ARMS fragment, and washed at a final stringency of 0.1X SSC at 60°C for 35 minutes. Emulsion-dipped slides were exposed for various times (days to weeks) prior to developing and then counterstained with thionin.

### Cell culture

[0078] Human embryonic kidney (HEK) 293 and 293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine. Native PC12 cells, TrkA-overexpressing PC12 cells (615) (Hempstead et al., 1992), and TrkB-overexpressing PC12 cells were maintained in DMEM containing 10% FBS and 5% heat-inactivated horse serum with 30 U/ml penicillin, 30 µg/ml streptomycin, 2 mM glutamine, and 200 µg/ml G418.

[0079] SCG neurons were prepared from P2 rats and cultured on collagen-coated coverslips in C-medium (minimum essential medium containing 10% fetal bovine serum (FBS) supplemented with 0.4% glucose and 2 mM L-glutamine) with 150 ng/ml 2.5S NGF (Harlan, Indianapolis, IN). To inhibit growth of non-neuronal cells, neurons were cultured in the presence of 24.6 µg/ml 5-fluoro-2-deoxyuridine and 24.4 µg/ml uridine.

[0080] Dissociated primary cultures of hippocampal neurons from embryonic day 17 (E17) rats were prepared from timed-pregnant Sprague-Dawley rats following published procedures (Aibel et al., 1998). Following dissection of the hippocampus, the meninges were removed. The tissue was briefly minced with fine forceps and then triturated through a fire polished pasteur pipet. Cells were counted and plated in Neurobasal media supplemented with B27 (Gibco, Gaithersburg, MD) on cell culture dishes coated overnight with 0.01 mg/ml poly-D-lysine. Cells were grown in a humidified incubator with 5% CO<sub>2</sub> at 37°C.

### Antibodies

[0081] To characterize the expression of the ARMS protein, polyclonal antibody against the carboxy terminus of ARMS was generated. A bacterially expressed GST fusion protein with the carboxy terminal 180 amino acids of ARMS was purified and used as antigen to generate rabbit antiserum (Cocalico Biologicals, Reamstown, PA). The specificity of this antiserum was determined by Western blot analyses of HEK293T cells transfected with a FLAG-tagged, full-length cDNA of ARMS. Lysates of transfected HEK293T cells, but not untransfected cells, displayed a specific 190 kilodalton species after immunoblotting with either an anti-FLAG antibody or anti-ARMS serum, 892.

# Transfection of mammalian cells, immunoprecipitation and immunoblotting

5 [0082] For transient transfection experiments, HEK293 or HEK293T cells plated at 70-80% confluency in 10 cm dishes were subjected to calcium phosphate transfection with different combinations of the mammalian expression plasmids containing cDNAs for ARMS, rat TrkA, and rat HA-tagged p75 (Khursigara et

10 al., 1999). PC12 615 cells or transiently transfected cells were harvested and lysed in 1 ml TNE buffer (10 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, and 1% NP40) containing 0.12 mg/ml phenylmethylsulfonyl fluoride (PMSF), 2 µg/ml leupeptin, 1 µg/ml aprotinin, 10 mM sodium fluoride (NaF), and 1 mM sodium

15 orthovanadate ( $\text{Na}_3\text{VO}_4$ ) for 30 min on ice. Following centrifugation and removal of the insoluble fraction, protein concentration of the supernatant was determined by the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as the standard. Cell lysates of equivalent

20 protein content were incubated for 4 hours to overnight with rotation at 4°C with either anti-pan-Trk polyclonal antibody, C14 (1.5 µg) or anti-ARMS (892) antiserum (1:100). The immune complexes were immobilized on protein A-Sepharose beads (Sigma), washed six times with ice-cold TNE buffer, boiled in SDS-sample

25 buffer, separated by sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE), and transferred onto PVDF membrane (Millipore, Bedford, MA). Immunoblotting was carried out by first blocking membranes in TBST buffer (20 mM Tris (pH 7.5), 500 mM NaCl, 0.1% Tween-20) containing 5% BSA for pY99 and 5% nonfat milk for others, and then incubating for 2 hours at room temperature or overnight at 4°C in TBST buffer containing one of the following primary antibodies: anti-Trk 44 serum (1:2000); anti-phosphotyrosine pY99 antibody (0.1 µg/ml) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-ARMS (892) antiserum (1:1000). Membranes were washed with TBST buffer and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit (Roche Molecular Biochemicals, Indianapolis, IN) or goat anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:10,000 and 1:7500 dilutions, respectively. Immunoreactive protein bands were detected by enhanced chemiluminescence using ECL reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

#### **Immunofluorescence analysis**

[0083] SCG neurons were fixed with 4% paraformaldehyde and blocked with PBS containing 0.075% saponin and 10% FBS or normal goat serum (NGS). Cells were then incubated with anti-ARMS (892, 1:1000) or anti-Trk B-3 antibody (1 µg/ml) in blocking buffer. Primary antibodies were visualized using fluorescence-conjugated

secondary antibodies (Cy3-conjugated goat anti-mouse IgG (Jackson Lab; 1:200) or FITC-conjugated goat anti-rabbit IgG (Jackson Lab; 1:100)). Images were collected on a BioRad confocal microscope.

5

## RESULTS

### Cloning of Ankyrin Repeat-rich, Membrane Spanning (ARMS)

[0084] Neurotrophins exert many biological activities, but few signaling molecules have been found to be specific for neurotrophin receptor signaling. The majority of proteins that serve as substrates for neurotrophin receptors are utilized by other receptor families. Effects on cellular and axonal migration, nerve regeneration, and apoptosis have been implicated for the p75 neurotrophin receptor, and effects on long term potentiation and synaptic transmission have been attributed to the Trks. To define unique molecules in neurotrophin signaling, a two-hybrid screen was undertaken using a rat dorsal root ganglion library as prey and the cytoplasmic domain of p75 as bait. This report describes a p75-interacting protein that is a downstream target for Trk signaling.

20 [0085] A positive cDNA clone was identified that encoded the carboxy terminal portion of a novel 1,715 amino acid protein. The predicted amino acid sequence contained eleven contiguous, 33 amino acid ankyrin repeats in the N-terminal domain and four

putative membrane-spanning regions. This protein was designated as ARMS for Ankyrin Repeat-rich, Membrane Spanning. The overall topology of ARMS is decidedly different from other transmembrane proteins. Other distinguishing features of this protein include  
5 a SAM domain, a polyproline stretch, and a potential PDZ-binding motif (Figures 1 and 2). These motifs represent candidate protein-protein interaction domains.

[0086] An extensive search of GenBank databases for proteins similar to ARMS identified many ankyrin-containing proteins;  
10 however, proteins sharing homology with ARMS in regions outside of the ankyrin repeats were notably absent. Analysis of *C. elegans* and *Drosophila* databases revealed ARMS orthologs in these organisms. Further analysis of human databases allowed the present inventors to determine the sequence of human ARMS from  
15 two overlapping ESTs in the database. The presence and conservation of ARMS sequences from nematodes to humans suggests that this protein may serve evolutionarily conserved functions. Between rat and human, the amino acid identity is 91% and similarity is 94% (Figure 2). Comparison of ARMS sequences among  
20 human, rat, *Drosophila* and *C. elegans* revealed similarity in overall structure: multiple ankyrin repeats in the amino terminus, four putative transmembrane domains, SAM domains, and consensus PDZ-binding motifs at the carboxymost three amino acids ("SIL" in human and rat; "TKL" in *Drosophila*, and "SDA" in *C.*



*elegans*). Despite the relatively divergent carboxy termini among the ARMS homologs, the presence of potential PDZ recognition sequences in all of these proteins indicates that conserved protein-protein interactions for ARMS may exist.

5 [0087] Interestingly, the regions most highly conserved were not the transmembrane (TM) domains, but certain regions surrounding them, namely the ankyrin repeats, the amino terminal portion between the ankyrin repeats and the first TM domain, the "loop" region between TM domains 2 and 3, and several stretches  
10 of amino acids in the carboxy terminus downstream of TM 4, including the SAM domain (Figure 3). The regions highlighted in Figure 3 range between 20% to 42% identical and 56% to 77% similar, whereas the TM domains are only 1% identical and 43% similar. It is interesting to note that embedded in these  
15 subdomains are conserved tyrosine residues; these potential phosphorylation sites are conserved among all four organisms examined (Figure 3, part 1). Although mutations in *C. elegans* and *Drosophila* ARMS have not yet been described, the *C. elegans* homolog, F36H1.2, is most similar to the ankyrin-related gene,  
20 UNC-44 (28%), which has a role in axon guidance, axonogenesis, and neuronal development (Otsuka et al., 1995).

### Distribution of ARMS transcripts in rat

[0088] In rat tissue, expression of ARMS mRNA was assessed by Northern blot analysis using a probe directed against the membrane-spanning regions of ARMS (Figure 4A). A single transcript of approximately 7.0 kilobases was detected. Although ARMS mRNA could be detected in several non-neuronal tissues, it was most abundant in the nervous system.

[0089] The expression of ARMS by *in situ* hybridization in adult rat brain was examined. Several populations of neurons were found to express ARMS mRNA, including mitral cells and cells of the glomerular layer of the olfactory bulb, all regions of the hippocampus, Purkinje cells of the cerebellum, and grey matter (most notably in presumed large motor neurons) of the spinal cord (Figures 5A-5D). One shared property of these neuronal populations is their ability to undergo synaptic changes throughout adulthood.

[0090] During defined periods of rodent embryonic development, subpopulations of sensory neurons require distinct neurotrophins and their cognate receptors for survival (Pinon et al., 1996). The neurotrophin receptor system remains functional through adulthood. Correspondingly, ARMS expression coincides with that of the Trks and p75. As shown by Northern analysis (Fig. 4A) and *in situ* hybridization (Figs. 6A-6B), ARMS expression persists in

the adult DRG. In addition to an absence of silver grains over the nerve fibers, there was a notable absence of ARMS message in a subset of DRG cell bodies corresponding to large diameter neurons. Although ARMS mRNA can be detected in all populations  
5 of DRG neurons, much lower expression in large diameter neurons suggests a less prominent role in proprioception.

[0091] More striking was the restricted expression of ARMS during development. In general, during rat nervous system development, the period of embryonic growth between E11 to E14 is  
10 a time of massive proliferation of the neuroepithelium. By E14, the first sets of postmitotic neurons are undergoing differentiation. In E14 rat embryos, ARMS was expressed in both spinal cord and dorsal root ganglia (Fig. 7A). These neuronal populations are among the first in the nervous system to undergo  
15 differentiation, and it is during this window of development that these postmitotic neurons are actively seeking and making connection with their targets. Additionally, several neuroanatomical loci of the developing brain expressed ARMS mRNA. In the midsagittal plane, the hippocampus, cortex, pons, and  
20 medulla were positive for ARMS expression (Fig. 7B). Regions lateral to the midline such as the basal telencephalon, superior/inferior colliculus, principal trigeminal sensory nucleus, and multiple ganglia, including trigeminal, geniculate,

vestibular, and superior cervical (Fig. 7C) showed significant levels of ARMS transcripts.

#### Association of ARMS with NGF receptors

5 [0092] Since the ARMS protein was originally identified in a yeast two-hybrid assay from its association with the cytoplasmic domain of the p75 receptor, the laboratory of the present inventors tested whether ARMS was capable of an interaction with p75 in cultured cells. HA-tagged p75 cDNA and the ARMS cDNA were  
10 co-transfected in HEK293T cells. After immunoprecipitation with an antibody (892) made against the C-terminal region of the ARMS protein, the p75 receptor could be readily detected by Western analysis (Figs. 8A and 8B). This transfection experiment indicated that ARMS and p75 are capable of forming a complex in  
15 HEK293T cells.

[0093] Previous studies indicated that p75 and the TrkA receptor can exist in a complex (Huber and Chao, 1995; Gargano et al., 1997; Bibel et al., 1999). To establish whether an interaction between TrkA and ARMS occurs, an immunoprecipitation  
20 experiment in PC12 cells expressing elevated levels of TrkA was carried out (Hempstead et al., 1992). An NGF-dependent association between ARMS and the TrkA receptor was detected following immunoprecipitation of TrkA and immunoblotting with the anti-ARMS antibody, 892 (Fig. 9A). The association between ARMS

and TrkA persisted 25 hours after NGF treatment. The endogenous association of TrkA and ARMS indicates that ARMS may exist in a ligand-dependent complex with Trk receptors.

[0094] To investigate further the distribution and potential  
 5 co-localization of ARMS and Trk in neuronal cells, indirect immunofluorescence experiments in primary cultures of rat sympathetic neurons. Using the 892 antibody against ARMS (Fig. 9B) and a monoclonal antibody directed against Trk (Fig. 9C), co-localization of ARMS and TrkA on the cell surface of sympathetic  
 10 neurons was observed (Fig. 9D, arrow). These results support the co-immunoprecipitation studies that suggest a physical association between Trk and ARMS.

#### Tyrosine phosphorylation of ARMS by NGF

15 [0095] The high degree of correspondence in the expression of ARMS and neurotrophin receptors and the endogenous association with TrkA receptors led the present inventors to postulate that ARMS might function as a target for Trk receptor phosphorylation. To investigate this possibility, cell lysates from NGF-treated  
 20 PC12 cells were prepared and the ARMS protein was immunoprecipitated with anti-ARMS antiserum, 892. Phosphorylation of ARMS protein was visualized by immunoblotting with an anti-phosphotyrosine antibody, pY99. Tyrosine phosphorylation of ARMS was detectable within a minute of NGF

addition (Figure 10A, top), closely following the time course of TrkA autophosphorylation (Figure 10A, bottom). With continued NGF treatment, the phosphorylation of ARMS persisted for 25 hours (Figure 10B). Pretreatment with the alkaloid-like compound K-252a (100 nM), which specifically inhibits NGF-mediated activity by selectively blocking the kinase activity of TrkA (Koizumi et al., 1988; Berg et al., 1992), completely abolished the tyrosine phosphorylation of ARMS. Hence, the ARMS protein represents a novel downstream target for the TrkA receptor tyrosine kinase.

[0096] To explore the specificity of ARMS phosphorylation, the effects of EGF and NGF were compared in PC12 cells. The phosphorylation of ARMS was considerably more robust following NGF treatment (Figure 11), even though both TrkA and EGF receptors were activated. These data indicate that ARMS phosphorylation by TrkA was a specific consequence of NGF, but not EGF, receptor signaling. This may be a consequence of a lack of association of ARMS with the EGF receptor. It is unlikely that ARMS phosphorylation was mediated through the MAP kinase pathway, since treatment of PC12 cells with EGF showed minimal tyrosine phosphorylation of ARMS after 10 minutes or 2 hours (Figure 11). Also, the pronounced ARMS phosphorylation was not elicited by other growth factors, such as insulin, FGF and insulin growth factor-1, in PC12 cells (data not shown),

indicating unique proteins are phosphorylated by the receptor tyrosine kinase, TrkA.

### Response to other trophic factors and ephrins

5 [0097] To test the ability of other Trk receptors to phosphorylate ARMS, PC12 cells stably expressing the TrkB receptor were used. Upon treatment of these cells with BDNF (100 ng/ml), ARMS was tyrosine phosphorylated, demonstrating that ARMS can be phosphorylated by TrkB as well as TrkA (Figure 12). That  
10 expression of ARMS is localized to some central regions in which TrkA is not expressed, such as the hippocampus, can be reconciled by the presence of TrkB, which is widely expressed in the adult CNS and which serves as a receptor for BDNF and NT-4/5 (Farinas et al., 1998).

15 [0098] To verify the ability of BDNF to induce phosphorylation of ARMS in neurons, primary cultures of hippocampal neurons were established. Hippocampal neurons express the TrkB receptor. After treatment with BDNF, ARMS was immunoprecipitated from hippocampal lysates and assessed for tyrosine phosphorylation by  
20 immunoblotting with an anti-phosphotyrosine antibody. A similar phosphorylation of ARMS after BDNF treatment in hippocampal neurons was observed (Figure 13). Thus, in a primary neuronal culture, ARMS is a downstream target of TrkB after activation by BDNF.

[0099] The expression of ARMS during developmental periods of axon outgrowth and synaptogenesis raises the possibility that ARMS is downstream of other known axon guidance regulators. The ephrin family was therefore examined because of its well-

5 established *in vitro* and *in vivo* guidance function. As seen in Figure 14A, the ARMS protein is potently tyrosine phosphorylated by ephrin B2 in a neuronal/glioma hybridoma cell line, NG108-15, that stably expresses EphB2 receptor. EphB2 receptor autophosphorylation and activation occurs over a time period of

10 30-40 minutes (Holland et al., 1997), in contrast to a much more rapid activation for Trks. Significantly, the phosphorylation of ARMS followed a time course similar to the time course of Eph receptor autophosphorylation (Figure 14B).

[00100] Unlike the Trk receptors, which are activated upon

15 dimerization by their respective ligands, Eph receptors are aggregated into multimeric complexes to be biologically active. This differential property of receptor aggregation may account for the differential time courses observed between neurotrophins and ephrins. Interestingly, the time course of TrkA and EphB2

20 phosphorylation of ARMS suggests that ARMS may be phosphorylated directly by the kinase domains of the receptors themselves and not via downstream intermediates. The integral membrane nature of ARMS and its potentially close proximity to receptor tyrosine kinases could facilitate such a process. Most important, these



results suggest that ARMS is phosphorylated by receptor tyrosine kinases with established roles in axonal targeting and guidance. It remains to be determined whether ephrin-Eph clustering and subsequent ARMS phosphorylation is involved in attractive or repulsive effects during axon guidance.

## DISCUSSION

### **ARMS: a novel downstream target for receptor tyrosine kinases**

[00101] Although the signaling properties of the Trk receptor tyrosine kinases have been studied extensively, there remain many neurotrophin-stimulated activities in which molecular mechanisms have not been fully defined. These include internalization and transport of receptors, growth cone guidance, and axonal and dendritic branching. A number of common substrates, including phospholipase C- $\gamma$ , PI-3 kinase, and Shc and Grb2 adaptor proteins, are utilized by many receptor tyrosine kinases, raising the question of how phosphorylation events lead to different biological outcomes (Chao, 1992b). One possibility is that there remain unique substrates that determine the specific nature of neurotrophin responses.

[00102] Here, the properties of a novel tyrosine phosphorylated transmembrane protein are described. The ARMS protein is highly expressed in many neuronal populations and functions as a downstream target for both Trks and Eph receptor.

A common feature of ARMS, p75, TrkA, and Eph receptors is that they all contain C-terminal PDZ-binding motifs. It is therefore possible that these proteins are localized to the same subcellular compartment by a PDZ-containing molecule, and this  
5 localization may contribute to signaling events both developmentally and in adulthood. Indeed, the EphB2 receptor has been found to be associated with PDZ-containing proteins, and this association is thought to be important for receptor function at neuronal synapses (Torres et al., 1998) and for vestibular  
10 axon guidance and ionic homeostasis in the inner ear (Cowan et al., 2000).

[00103] At E14, trigeminal and vestibular axons migrate toward their peripheral targets and commence synapse formation. High levels of ARMS mRNA expression in these subpopulations  
15 suggest that ARMS may participate in axonogenesis or axon guidance. A notable absence of ARMS mRNA expression was observed in germinal zones of the developing brain, regions that are extensively proliferating. A general conclusion that can be drawn from the *in situ* hybridization studies described here is  
20 that ARMS-positive neuron populations, for the most part, are post-mitotic and post-migratory. For example, the somatic and visceral motor neurons of the spinal cord are born by E12 and E13, but by E14 are already undergoing axonogenesis (Paxinos, 1995). It is during this post-migratory, differentiative stage

that ARMS is highly expressed. Conversely, the presumptive olfactory bulb, which is devoid of neurons at this stage of development, is negative for ARMS expression; the absence of ARMS expression in pre-migratory neuronal populations supports the observation that ARMS expression may be restricted to post-mitotic and post-migratory neurons. These findings suggest that ARMS may be involved in post-migratory events, such as axon guidance or synaptogenesis.

[00104] Expression of ARMS in the adult central nervous system was observed in regions such as the olfactory bulb, hippocampus, Purkinje cells of the cerebellum, and spinal cord motor neurons. The most significant commonality of these neuronal populations is their ability to undergo continued synaptic changes throughout adult life. Neurons of the olfactory bulb are continually renewed and hence must form new synapses; hippocampal neurons can undergo synaptic remodeling and LTP; Purkinje cell dendrites are highly plastic due to their constant structural remodeling; and motor neurons have the capacity to regenerate and to form new synapses with peripheral targets in adults. Peripheral neurons have regenerative properties throughout adulthood, and this process requires axon outgrowth and new synapse formation. Neurotrophins and ephrins are likely candidates in spinal cord regeneration (Frisen et al., 1992;

Miranda et al., 1999), and the present inventors believe that ARMS is also utilized in this process.

# **Colocalization of ARMS with neurotrophin and ephrin receptors**

5        [00105]        The *in vivo* utilization of ARMS by the neurotrophin and ephrin receptors is supported by co-expression of these proteins during development. For example, Trk and p75 expression can be detected in all peripheral ganglia of neural crest origin (e.g., superior cervical and dorsal root), spinal  
10    cord, and brainstem, regions also high in ARMS mRNA (Yan and Johnson, 1988; Snider, 1994). In the adult, ARMS and p75 expression overlap most notably in the olfactory bulb, Purkinje cells of the cerebellum and motor neurons of the spinal cord. Additionally, Trk receptors are found throughout the nervous  
15    system in regions that also express ARMS. For example, TrkA and ARMS are both expressed in the small diameter sensory neurons of the DRG (Averill et al., 1995; Wright and Snider, 1995). Adult hippocampus, cerebellar Purkinje cells, and motor neurons of the spinal cord are a few regions in which the expression of ARMS and  
20    TrkB significantly overlaps (Yan et al., 1997).

      [00106]        In a similar manner, Eph receptor and ARMS expression overlap in adult hippocampus and spinal motor neurons. During development, the Eph receptors are also found in ganglia of neural crest origin (e.g., sensory and vestibular) and in the

5 tectum (Flanagan and Vanderhaeghen, 1998), regions that also express ARMS. Ephrins and their receptors have a well known function in establishment of the retinotectal pathway, and the expression of ARMS in the tectum suggests that ARMS plays a role in this process.

[00107] Given the multiple cell populations that express Trks, Eph receptors and ARMS, such as sensory and motor neurons, our findings raise the possibility of cross-talk between these two receptor systems. ARMS may serve to link or modulate these two signaling pathways in a cooperative or competitive manner. This could potentially be achieved by receptor phosphorylation of similar or different tyrosine residues of ARMS and/or association with other membrane proteins. It is plausible that ARMS may serve as an adaptor protein, given its PDZ-binding motif and other protein interaction domains that would allow for recruitment of multiple, diverse proteins.

## EXAMPLE 2

### MATERIALS AND METHODS

#### 20 Yeast two-hybrid screening

[00108] The two hybrid interaction screenings were performed using 3 different baits named 2.1, 7 and 10 that corresponded to amino acids S1303-L1715, D1056-S1151 and L1603-L1715 of ARMS protein (SEQ ID NO:2), respectively. The bait

plasmids were generated by PCR with rat ARMS cDNA and the amplified fragments were ligated into pEG202 at *Bam*HI-*Nco*I sites as an in-frame fusion with the LexA-DNA binding domain.

[00109] A cDNA library from postnatal day (P1) dorsal root ganglia (DRG) was generated (see Example 1). The DRG library  
5 cDNAs were expressed as in-frame fusions with the Gal4 transcriptional activation domain. Approximately  $5 \times 10^7$  yeast transformants were screened for growth in absence of leucine. Positive-interacting clones were confirmed with  $\beta$ -galactosidase  
10 activity and their specificity was tested using unrelated proteins as baits.

## RESULTS

[00110] As demonstrated in Example 1, Figures 15A and 15B  
15 show that ARMS interacts with TrkA, TrkB, and TrkC receptors but not with EGF receptors.

[00111] The laboratory of the present inventors have also found that following phosphorylation, ARMS becomes localized discretely at growth cones and in synaptic regions of neuronal  
20 membranes as visualized using anti-ARMS antibodies in immunofluorescence studies. Figure 18 is an immunofluorescence analysis that shows ARMS localized in growth cones and axons of hippocampal neurons. It was further demonstrated that ARMS co-

localizes with Vamp-2, a synaptic vesicle marker, in hippocampal neurons (Figs. 17A-17F).

[00112] The results of immunofluorescence and confocal microscopic studies show that there is a dramatic change in distribution of ARMS in both hippocampal neurons and PC12 cells after treatment with BDNF or NGF (Figs. 16A and 16B), indicating that ARMS becomes concentrated at synaptic sites where neuronal activities are located.

[00113] The laboratory of the present inventors further identified several neuronal proteins (Table 1) that interact/bind with the ARMS protein using a yeast two-hybrid screening system.

**TABLE 1: Proteins Interacting with ARMS**

PROTEIN	LOCALIZATION AND FUNCTION	REFERENCES
Cell division control related Protein 1 (CDCrel-1)	Predominantly expressed in the brain and associates with membranes including synaptic vesicles. Inhibition of exocytosis	Beites et al., 1999
Calcium channel beta-3 subunit	Predominantly expressed in the brain but also in aorta, lung, heart, pancreas and the adrenal gland. Regulation of $Ca^{2+}$ entry into the cell	Castellano et al., 1993
Actin related protein 2/3 complex, subunit 3 (21 kDa) (ARPC3)	Part of a complex implicated in the control of actin polymerization in cells.	Welch et al., 1997
Synembryn	Regulation of neurotransmitter secretion by controlling the production and consumption of diacylglycerol.	Miller et al., 2000
GRIP1 and GRIP2 (ABP)	enriched in brain synapses  Possible role in either an anchoring of AMPA receptors at synapses or in the trafficking of AMPA receptors	Dong et al., 1997 Srivastava et al., 1998

The proteins in Table 1, identified as interacting with ARMS, include several synaptic vesicle proteins, a calcium channel subunit and proteins involved in clustering glutamate channels (NMDA, kainate). These interactions suggest that the ARMS protein is involved in neurotransmitter vesicle movements in nerve cells, in the release of neurotransmitters, i.e., acetylcholine or dopamine, and in the clustering of ion channels, all of which cause changes in neuronal activity (action potentials).

[00114] There is growing evidence that neurotrophins, such as NGF and BDNF, affect neurotransmitter release and neuronal activity, in addition to promoting neuronal survival (Auld et al., 2001; Albeck et al., 1999; Pozzo-Miller et al., 1999; Jia et al., 1999; Sala et al., 1998; Kang et al., 1995; Korte et al., 1995; Toledo-Aral et al., 1995). The significance of these findings stem from the potent effects of neurotrophins upon synaptic plasticity. The phosphorylation of ARMS by Trk and Eph receptors provides a mechanism for transmitting information from neurotrophins to specific regions of synaptic activity in nerve cells.

[00115] PDZ proteins, such as GRIP1 and GRIP2 in Table, contain a protein interaction domain (PDZ) that bind in a sequence specific way to short C-terminal sequences in a wide variety of membrane proteins. The function of PDZ proteins is to



assemble complexes of proteins that perform signaling functions in specific subcellular locations, such as at synapses or growth cones. PDZ-containing proteins have been shown to cluster AMPA receptors, such as GluR2 and GluR3 (Dong et al 1997; Srivastava et al 1998). The Trk receptors were demonstrated to associate with ARMS (Example 1 and Figs. 15A and 15B). Furthermore, ARMS can recruit PDZ proteins such as GRIP1 and GRIP2 (Figure 19), which possesses seven PDZ motifs. This interaction between ARMS and GRIP proteins is most likely between the C-terminus of ARMS (SIL) and the first PDZ repeat of GRIP. The AMPA glutamate receptors bind to the 7<sup>th</sup> PDZ repeat of GRIP through its C-terminal sequence VKI and can also bind to the 3<sup>rd</sup>-6<sup>th</sup> PDZ repeats of GRIP.

[00116] These binding interactions have been extensively defined for many proteins (Sheng et al., 2001). The interactions between ARMS and GRIP proteins link ARMS to ion channels which can regulate neuronal activity. Since ARMS is a specific and robust substrate for Trk receptor phosphorylation, neurotrophins may therefore communicate with components of synapses through the ARMS and PDZ-containing proteins. This provides a potential mechanism to explain changes in synaptic plasticity that have been detected with neurotrophins (Thoenen, 1995 Poo, 2001).

[00117] Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

[00118] While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the inventions following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

[00119] All references cited herein, including journal articles or abstracts, published or corresponding U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by references.

[00120] Reference to known method steps, conventional methods steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

[00121] The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

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